Evidence for a step-wise program of T cell development within the human tonsil

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Abstract

Human T cell development within the thymus is a well-characterized event, and it is known that a functional thymus is required for normal T cell generation to take place. Individuals with poor thymic function, from either congenital abnormality or post-natal injury, have a difficult time mounting a proper immune response and suffer from debilitating immune deficiency. Thus, it would be highly beneficial to identify an extrathymic human tissue that can support T cell differentiation so that we may eventually augment T cell production in these individuals. However, to date, no definitive site of extrathymic T cell development has been established. In this study, we used flow cytometry to identify five populations of putative extrathymic T cell precursors within the human pediatric tonsil. These populations range from an early $CD34^{+}CD38^{dim}Lin^{-}$ subset all the way to a $CD1a^{+}CD4^{+}CD8^{+}CD3^{+/-}$ double positive pre-T cell. Each subset identified phenotypically resembles the same population found in the human thymus, suggesting that there is considerable similarity between the cells in each of these two tissues. Furthermore, each putative extrathymic T cell developmental intermediate found within the tonsil retains the ability to develop into a mature $CD3^{+}$ T cell. Thus, our data suggest that the human tonsil contains a full spectrum of T cell developmental intermediates that are remarkably similar to those found in the human thymus, and that the tonsil may be an extrathymic site which supports complete T cell differentiation.

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Introduction

Human hematopoiesis is the process whereby hematopoietic stem cells (HSCs) and progenitor cells (HPCs) give rise to all cells of the immune system. After birth HPCs within the thymus specifically give rise to T cells, critical mediators of the adaptive immune system[1]. Indeed, a functional thymus is necessary for the development of a complete T cell repertoire in a normal individual, and individuals sustaining thymic malformation or injury are prone to immune deficiency[2]. However, whether non-thymic tissues can support T cell development is currently unknown. Our laboratory has previously published that HPCs expressing CD34, a marker of immaturity, reside within the human tonsil and lymph node, and we have established that as a bulk population these cells have the ability to differentiate into T cells, natural killer cells, and dendritic cells in *ex vivo* assays[3,4]. These data suggest that it is possible that a hematopoietic progenitor cell capable of T cell differentiation seeds these secondary lymphoid tissues, but whether the tonsil or lymph node microenvironment can actually support full T cell development has yet to be established.

Human T cell development takes place through a well-characterized set of cellular stages, each of which is defined by a unique expression of antigens on the cell surface[1]. Thymic T cell development begins with an immature cell called the Thymic Seeding Progenitor (TSP), which is phenotypically characterized as $CD34^{+}CD38^{dim}Lin^{-}$. This cell is a multipotent, highly replicative progenitor cell, and represents the first thymocyte which displays signs of T cell receptor (TCR) gene rearrangement[1,5]. Soon after entry into the thymus, the TSP increases the expression of CD38 on its surface, yielding a slightly more mature $CD34^{+}CD38^{bright}Lin^{-}$ cell, which then gains acquisition of the marker CD1a. The resulting $CD34^{+}CD1a^{+}$ population, known as a pre-T cell, is

the first thymic subset to show signs of developmental commitment to the T cell lineage[1]. Next, the thymocyte loses the expression of CD34 as it increases the expression of CD4 and CD8 on its cell surface, to form what is ultimately known as the $CD4^+CD8^+$ double positive (DP) pre-T cell. The DP cell expresses CD1a, is committed to the T cell lineage, and begins to express variable levels of the T cell specific marker CD3[1]. Upon acquisition of CD3, the thymocyte downregulates expression of either CD4 or CD8 and begins to express a functional T cell receptor to become a single positive naïve T cell[1].

While this process has been well defined in the thymus, whether or not it can take place extrathymically is currently unknown. The question of whether or not extrathymic tissues can and do support T cell development is clinically significant. Should a secondary lymphoid tissue be able to host complete T cell differentiation, it is possible that future methods could be developed to augment and enhance this phenomenon in the situation of poor thymic function. Thus, this study sought out to establish if the human tonsil does contain a pathway of extrathymic T cell development in normal physiological conditions.

Results and Discussion

In this study, we obtained fresh human tonsils from pediatric elective tonsillectomy patients and used flow cytometry to investigate whether or not the bulk population of tonsillar cells contains extrathymic T cell precursors. A single cell suspension was made from human tonsillar cells, and the resulting mononuclear fraction was enriched for cells expressing the marker CD34. Within the CD34-enriched tonsillar subset, we first identified a $CD34^+CD38^{dim}$ and a $CD34^+CD38^{bright}$ population neither of which expresses markers specific to any mature human hematopoietic lineage (Lin^-) (Figure 1A). Phenotypically, these cells resemble the thymic seeding progenitor and the more mature downstream $CD34^+CD38^{bright}Lin^-$ cells identified in the

thymus. Thus, this data suggested that perhaps the human tonsil contains early precursor cells, which resemble those of the human thymus.

Next, we sought to determine if the tonsil contains pre-T cells similar to those found in the thymus. CD34-enriched tonsillar cells were analyzed for the expression of CD1a, which is found on both pre-T cells[1] and on antigen presenting cells (APCs)[6,7]. Within the total $CD34^+$ subset, the tonsil contains a small but consistent population of $CD34^+CD1a^+$ cells, which upon further analysis, expresses either CD10 or CD11c (Figure 1B). As CD10 is consistently found on thymic $CD34^+CD1a^+$ pre-T cells[1], and CD11c is a marker APCs that is not found on thymic pre-T cells (data not sown), we hypothesize that the $CD34^+CD1a^+CD10^+CD11c^-$ fraction of the human tonsil represents an extrathymic pre-T cell, whereas the similarly identified population expressing CD11c, may be a distinct lineage altogether.

The data presented in Figure 1 highly suggests that the earliest stages of T cell development, including a $CD34^+CD38^{dim}Lin^-$ multipotent progenitor, a $CD34^+CD38^{bright}Lin^-$ precursor, and a $CD34^+CD1a^+CD11c^-$ pre-T cell may reside within the human tonsil. To further establish whether or not this is true, we examined the expression of more than 25 human hematopoietic markers on the surface of these three subsets from both the tonsil and the thymus Total mononuclear cells from each tissue were enriched for CD34-expressing cells, and flow cytometry was used to gate on the $CD34^+CD38^{dim}Lin^-$, $CD34^+CD38^{bright}Lin^-$, or $CD34^+CD1a^+CD11c^-$ subsets from each tissue (Figure 2A-B).

The $CD34^+CD38^{dim}Lin^-$ and $CD34^+CD38^{bright}Lin$ tonsillar subsets shared many phenotypic characteristics with those from the thymus. For example, The $CD34^+CD38^{dim}Lin^-$ subsets from the tonsil and the thymus were similar in that both express CD10, low levels of CD25, CD33, CD45RA, and HLA-DR, and lack expression of CD4, CD8, CD56, and CD116.

Similarly, Similarly, $CD34^{+}CD38^{bright}Lin^{-}$ cells from both the tonsil and the thymus express CD2, CD10, and CD45RA, but lack expression of CD4, CD8, CD56, and CD116. However, these two subsets from the tonsil also demonstrate some differences from their thymic counterparts as seen in Figure 2. It is possible that these differences reflect that different progenitor cells seed the tonsil and the thymus, or alternatively that the microenvironment of each tissue differently effects the phenotype of the same progenitor upon its entry into either the tonsil or thymus. Future studies should address this issue.

Intriguingly, the $CD34^{+}CD1a^{+}CD11c^{-}$ populations from both the tonsil and the thymus are remarkably similar in phenotype. Both express CD2, CD7, CD5, CD10, CD127, and intracellular TdT. Similarly, both lack expression of CD8, CD25, CD116, CD56, CD33, and HLA-DR (Figure 2A-B). Importantly, a lack of CD116, CD33, and HLA-DR further confirms that this subset from the tonsil likely does not represent a cell that is part of an antigen presenting lineage. Furthermore, $CD34^{+}CD1a^{+}CD11c^{-}$ cells from both the thymus and the tonsil express variable levels of CD4, indicating that each is transitioning to an early $CD4^{+}$ single positive cell, typically seen at this stage of thymic development[1]. Overall, the data presented in Figure 2 suggest that the human tonsil contains early precursor cells that appear similar in phenotype to the first three stages of human T cell development, and that this extrathymic tissue is capable of supporting at least the earliest steps of T cell differentiation.

To establish if the human tonsil contains T cell developmental intermediates beyond these early $CD34^{+}$ precursors, we next sought to identify $CD34^{-}$ pre-T cells. In the thymus, $CD34^{+}CD1a^{+}$ pre-T cells lose CD34 as they increase the expression of CD4, CD8 and eventually CD3 on their surface, all the while retaining CD1a expression[1]. Thus, we used flow cytometry to examine the relationship of CD34, CD1a, and CD3 in the tonsil to see if a complete spectrum of

these populations may reside within this tissue. To do so, we simultaneously enriched CD19-depleted tonsillar cells for cells expressing CD34, CD1a, or a combination of these two markers.. The relationship between CD34, CD3, and CD1a suggests that as in the thymus, tonsillar $CD1a^{+}CD11c^{-}$ cells can be divided between those that express CD34, those that express neither CD3 nor CD34, and those that express CD3 (Figure 3). This is in line with a model wherein cells progress from a CD34-expressing precursor to a more mature $CD34^{-}CD3^{-}$ pre-T cell and finally to a more mature $CD3^{+}$ naïve T cell.

To further examine whether or not $CD34^{-}CD1a^{+}CD3^{+/=}$ tonsillar cells represent true extrathymic T cell precursors, we examined three $CD34^{-}CD11c^{-}$ populations in both the tonsil and the thymus for the expression of T cell-associated antigens: $CD34^{-}CD1a^{+}CD3^{-}CD11c^{-}$ cells, $CD34^{-}CD1a^{+}CD3^{+}CD11c^{-}$ cells, and $CD34^{-}CD1a^{-}CD3^{+}CD11c^{-}$ cells (Figure 4A-B). In both tissues, as the cells progressed from $CD1a^{+}CD3^{-}$ to $CD1a^{+}CD3^{+}$ and then to $CD1a^{-}CD3^{+}$, there was a consistent decrease in expression of the early T cell proteins TdT and CD10, but an increase in the expression of the mature T cell markers CD5, CD7 and in T cell receptors. Interestingly, in both tissues, a large majority of $CD34^{-}CD1a^{+}CD3^{-}CD11c^{-}$ cells expressed both CD4 and CD8 (Figure 4A-B), indicating that these cells likely represent the true $CD4^{+}CD8^{+}$ DP cells that are so well characterized in human thymic T cell differentiation.

The phenotypic data presented thus far suggests that five populations of putative extrathymic T cell precursors reside within the human tonsil: 1) $CD34^{+}CD38^{dim}Lin^{-}$; 2) $CD34^{+}CD38^{bright}Lin^{-}$; 3) $CD34^{+}CD1a^{+}CD3^{-}$; 4) $CD34^{-}CD1a^{+}CD3^{-}$; and 5) $CD34^{-}CD1a^{+}CD3^{+}$ cells (Figure 5A). To verify that each population identified could differentiate into mature T cells, we used fluorescence activated cell sorting (FACS) to isolate each subset to purity. As a control, we also sorted $CD34^{-}CD1a^{-}CD3^{+}$ cells (Population 6; Figure 5A), which represent the

majority of mature $CD3^+$ T cells in the tonsil. We then tested their T cell differentiation capabilities in a well established *ex vivo* culture system, wherein each population was cultured in the presence of OP9-DL1 cells and the cytokines Flt3 Ligand (FL) and Interleukin-7 (IL-7). Furthermore, we performed the same experiments from these six populations isolated from the human thymus for comparison. All six populations from both the tonsil and the thymus retained T cell developmental potential, as evidenced by the emergence of $CD3^+$ T cells that expressed variable levels of CD4, CD8, and T cell receptors on their surface after 26 days (Figure 5B-C). These data suggest that the five populations identified in this study have T cell potential, and that the tonsil contains a full spectrum of extrathymic T cell developmental intermediates similar to those found in the human thymus.

In summary, our phenotypic and differentiation data suggest that the human tonsil contains a spectrum of T cell developmental intermediates beginning with a $CD34^+CD38^{dim}$ hematopoietic progenitor cell which progresses to a $CD34^+$ pre-T cell and later a $CD34^-CD1a^+CD4^+CD8^+CD3^{+/-}$ T cell precursor. The identification of the tonsil as a site for extrathymic T cell development challenges the paradigm that the thymus is the only organ responsible for complete T cell genesis, and raises the possibility that other lymphoid organs could support T cell differentiation in the situation of poor thymic function resulting from injury or disease.

Experimental Procedures

Human Cell Isolation: Human tonsils and thymuses were obtained from pediatric donors at Nationwide Children's Hospital (Columbus, OH) under the approval of the Ohio State University Institutional Review Board and according to the guidelines set forth by the Declaration of Helsinksi. Single cell suspensions were made from both tonsils and thymuses by

manual homogenization and passing through a 70 μm mesh strainer. Total mononuclear cells were then obtained via density centrifugation on Ficoll-Paque Plus. To enrich tonsillar tissue for total $CD34^+$ cells, $CD3^+$ T and $CD19^+$ B cells were first depleted from the mononuclear cell fraction using magnetic microbead separation (Miltenyi Biotec). The resulting fractions were then enriched for CD34-expressing cells using an indirect CD34 isolation kit (Miltenyi Biotec). To enrich tonsillar cells for CD34 and CD1a-expressing cells, $CD19^+$ B cells were magnetically depleted from the total mononuclear cell pool, and the resulting cells were then simultaneously subjected to CD34 and CD1a magnetic enrichment (Miltenyi Biotec). Human thymic mononuclear cells were enriched for CD34-expressing cells using an indirect CD34 isolation kit (Miltenyi Biotec).

Flow cytometry and cell sorting: All fluorescently labeled antibodies used were purchased from BD Biosciences, Beckman Coulter, or Mitlenyi Biotec. Intracellular staining for TdT was performed after surface staining using the BD Cytofix/Cytoperm Plus fixation/permeabilization kit (BD Biosciences) according to the manufacturer's recommendations. Flow cytometry was performed on a FACS Vantage (BD Biosciences) or LSR II (BD Biosciences), and data was analyzed using FlowJo software (Treestar, Inc). Cell sorting of T cell precursors from the thymus or tonsil was performed on a FACS Aria (BD Biosciences). Purity assessments routinely revealed that sorted populations were >95% pure.

T cell differentiation Assays: T cell differentiation assays were performed in flat-bottom 96 well plates in the presence of OP9-DL1 stromal cells (a gift from J.C. Zúñiga-Pflücker, University of Toronto, Toronto, Canada), 100 ng/ml Flt3 ligand (FL), 10 ng/ml IL-7 (Miltenyi Biotec), and α-MEM plus L-glutamine (Gibco) supplemented with 20% fetal bovine serum, penicillin G (100 μg/ml) and streptomycin (100 μg/ml) (Gibco). Cultures were initiated with a

starting cell density of 450-5,000 hematopoietic precursor cells per well. Every 7-9 days, cells were disrupted, passed through a 70 μm cell strainer, and were deposited on new stromal cells with fresh cytokine-supplemented medium. In between these cell transfers, one half of the medium was removed every 4-5 days and was replaced with fresh medium containing 2x cytokines.

Figure 1

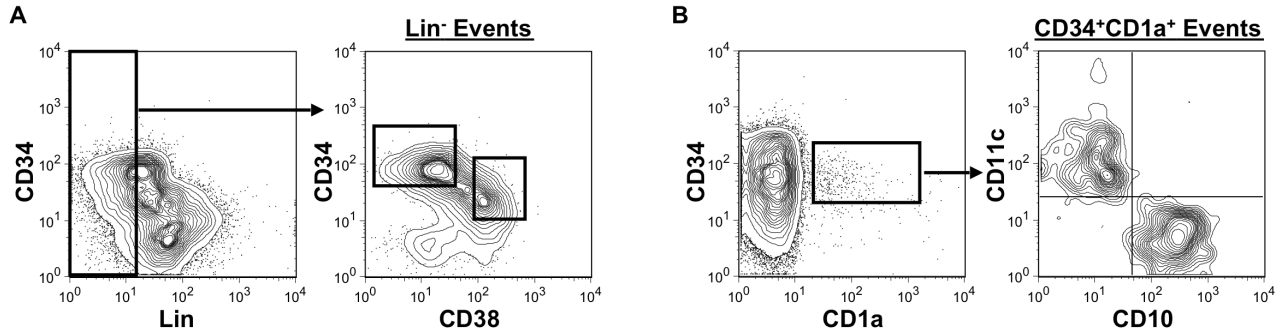

Figure 1: The human tonsil contains $CD34^{+}CD38^{dim}Lin^{-}$, $CD34^{+}CD38^{bright}Lin^{-}$, and $CD34^{+}CD1a^{+}CD11c^{-}$ cells. CD19 and CD3-depleted tonsil cells were magnetically enriched for CD34-expressing cells. (A) Expression of CD34 and the lineage markers CD11c, BDCA-2, CD117, CD161, CD19, CD3 and CD1a (Lin) on CD34 enriched tonsillar cells (left). Total lymphocytes were gated on Lin^{-} events and analyzed for their expression of CD34 and CD38 (right). The two rectangles illustrate the relative differences in CD34 surface density for the $CD34+CD38^{dim}$ ("dim") and $CD34+CD38^{bright}$ ("bright") subsets. (B) Expression of CD1a on CD34 enriched tonsillar cells (left). Events were gated on $CD34^{+}CD1a^{+}$ cells and analyzed for their co-expression of CD10 and CD11c (right).. All data are from a representative tonsil of >10 (A), or 4 (B) individual donors.

Figure 2

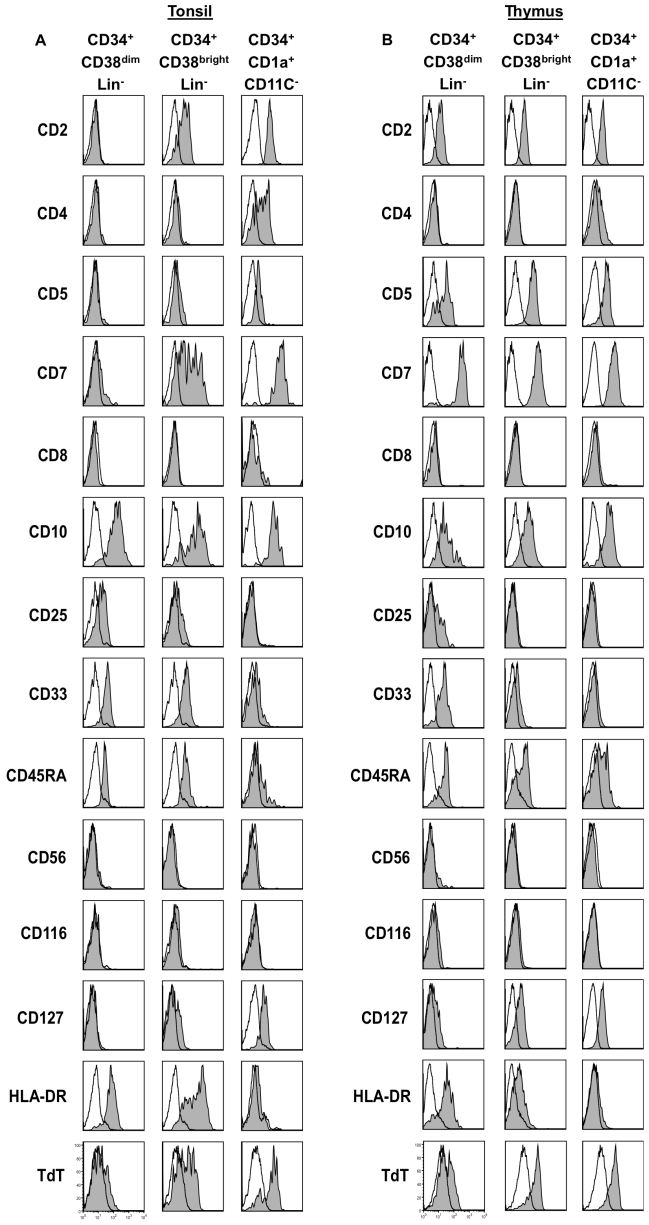

Figure 2: Phenotypic comparison of $CD34^+CD38^{dim}Lin^-$, $CD34^+CD38^{bright}Lin^-$, and $CD34^+CD1a^+CD11c^-$ cells in human tonsil and thymus. (A) Tonsillar mononuclear cells were magnetically depleted of CD3 and CD19 expressing cells, and were then enriched for $CD34^+$ cells. Enriched cells are gated on $CD34^+CD38^{dim}Lin^-$ (left), $CD34^+CD38^{bright}Lin^-$ (middle), or $CD34^+CD1a^+CD11c^-$ (right) events. (B) Thymic mononuclear cells were magnetically enriched for $CD34^+$ cells and then gated on the same three populations as in (A). Data for each antigen is from a representative donor.

Figure 3

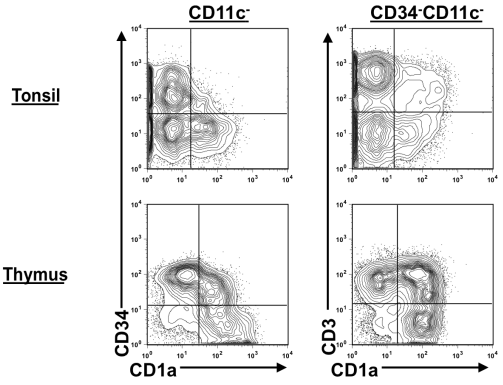

Figure 3: The relationship between CD34, CD1a, and CD3 within the human tonsil. CD19-depleted tonsillar cells were simultaneously enriched for both CD34 and CD1a-expressing cells. Enriched cells were gated on $CD11c^-$ (left) or $CD34^-CD11c^-$ (right) events and analyzed for expression of CD34 and CD1a (left), and CD3 and CD1a (right). Thymic $CD34^+$ and $CD34^-$ cells were similarly analyzed after CD34 magnetic selection. Data are from representative donors, and each analysis was performed in ≥3 independent tonsils, or thymuses.

Figure 4

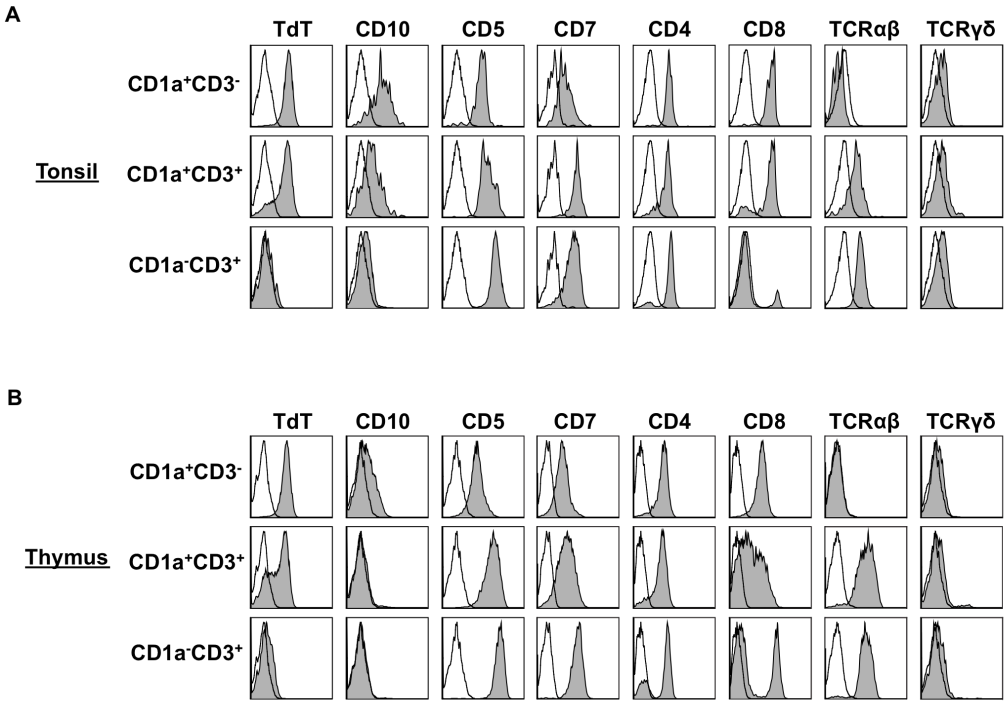

Figure 4: Putative extrathymic T cell precursors in the human tonsil acquire T cell associatedantigens in a fashion similar to those in the thymus. (A) CD1a-enriched tonsillar cells were gated on $CD34^{-}11c^{-}$ events and analyzed for the expression of T cell-associated antigens on three populations: $CD1a^{+}CD3^{-}$; $CD1a^{+}CD3^{+}$; and $CD1a^{-}CD3^{-}$ (B) CD34-depleted thymic cells were gated on the same three populations and analyzed as in (A). Data are from representative donors, and each analysis was performed in three independent tonsils or two independent thymuses. In each histogram, filled lines indicate staining with the indicated antibody, whereas open lines indicate staining with an isotype-matched control.

Figure 5

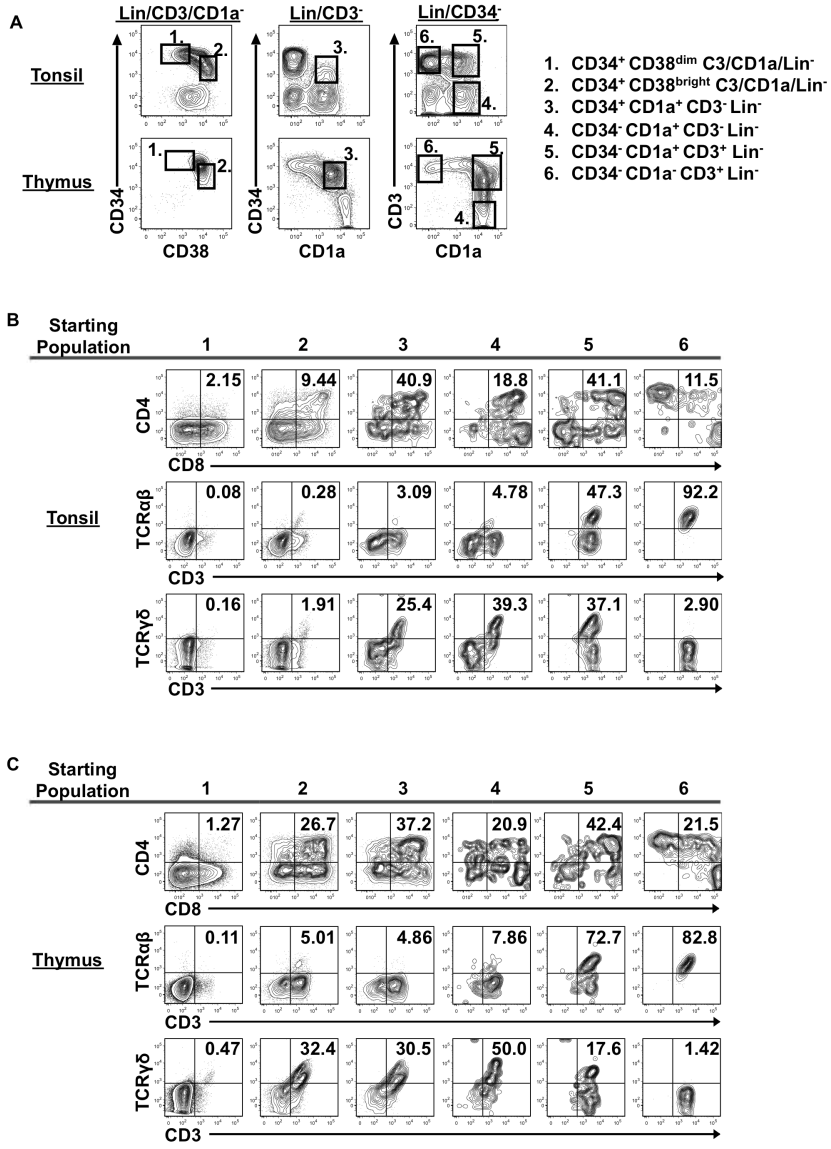

Figure 5: T cell differentiation potential of six putative extrathymic T cell precursors in the human tonsil. (A) Tonsillar mononuclear cells were depleted of $CD19^+$ events, enriched for $CD34^+$ and $CD1a^+$ cells, and the six populations shown were isolated using FACS. (B) All six populations were cultured independently on OP9-DL1 cells with FL and IL-7 for 26 days. After harvest, cells were analyzed for the expression of CD3, CD4, CD8, TCRαβ, and TCRγδ. Data

are gated on $GFP^{-}CD45^{+}$ events to exclude OP9-DL1 stromal cells from analysis. (C) Thymus mononuclear cells were magnetically enriched for $CD34^{+}$ events. Populations 1-3 were sorted from the $CD34^{+}$ fraction, and populations 4-6 were sorted from the $CD34^{-}$ fraction. Each population was cultured on OP9-DL1 cells with FL and IL-7 for 26 days. After harvest, cells were analyzed as in (B). Data shown is representative of 3 experiments performed with individual tonsil donors (B) or of 2 experiments performed with individual thymus donors (C). Numbers represent the mean percentages of $GFP^{-}CD45^{+}$ cells which stained double positive for both antigens indicated on the dot plot.

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